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THE ALLEGED ANTITOXIC ACTION OF VITAMIN C IN DIPHTHERIA.

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THE isolation and chemical identification of vitamin C (*l*-ascorbic acid) was followed by an indiscriminate search for possible functions of this interesting compound other than its antiscorbutic activity. Although evidence obtained before this period did not promise well, the bearing of vitamin C on various immunological reactions has also been receiving its ample share of attention during the last few years.

In some early experiments (Zilva, 1919) it was found that the serum of guinea-pigs suffering from chronic scurvy after immunization with *B. typhosus* yielded agglutination and amboceptor titres of the same order as that obtained from the serum of normally-fed animals similarly immunized. Nor did the chronic vitamin C deficiency influence the activity of the complement of sera from scorbutic guinea-pigs. In the case of diphtheria (Arkwright and Zilva, 1924 ; Bieling, 1925) it was observed that after intra- or subcutaneous injections of diphtheria toxin or living bacilli in guinea-pigs suffering from chronic scurvy, the inflammatory reaction was decidedly less than when the animals used were kept on a normal and well-balanced diet. That this was not due to the vitamin C deficiency *per se* was evident from the fact that animals subsisting on a quantitatively restricted diet which contained ample quantities of vitamin C showed similarly an attenuated local reaction. Bieling had observed at the same time that scorbutic guinea-pigs succumbed to diphtheria toxin more readily than normally-fed animals, but the disease was so far advanced in his animals at the time of injection that beaded costo-chondral junctions were found at autopsy, and consequently the lowered resistance to the action of the toxin cannot be justifiably ascribed to a specific vitamin C deficiency.

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The work of Harde and her collaborators (Harde, 1934; Harde and Philippe, 1934; Greenwald and Harde, 1935) was the first sign of returning interest in the subject of the influence of vitamin C on the susceptibility of the animal organism to diphtheria toxin. Impressed by the fact that in guinea-pigs which received diphtheria toxin, the adrenals suffered a diminution in their vitamin C content, whilst those of the mouse, which synthesizes the vitamin, and which was found to be resistant to 1–10 M.L.D. of diphtheria toxin, retained their full load of ascorbic acid, even after the injection of the toxin, they instituted experiments with the object of studying the action of ascorbic acid on diphtheria toxin *in vitro* and *in vivo*. In the former case it was observed that the addition of neutralized ascorbic acid had a destructive effect on diphtheria toxin, and although no clear-cut data were obtained *in vivo*, the results were considered by these workers to favour the view that vitamin C had a protective action against diphtheria toxin. In the same connection King and Menten (1935) found that when guinea-pigs were partially depleted of their vitamin C body-reserve without showing signs of scurvy, and injected with graduated doses of diphtheria toxin, their survival time was shortened by 50 p.c., and their loss in body-weight was greater than in the case of animals with higher stores of vitamin C. Jungeblut and Zwemer (1935) also concluded from their experiments *in vitro* and *in vivo* that vitamin C plays an important part in the mechanism of natural resistance to diphtheria toxin. It is of interest to note that these workers, unlike Arkwright and Zilva and Bieling, found that the storage of vitamin C by the guinea-pig had a pronounced effect in inhibiting or even suppressing the local reaction after intracutaneous injections of diphtheria toxin. Jeney, Gagyi and Baranyai (1936), too, claimed that a daily dose of 1–2 mg. of ascorbic acid imparted higher resistance to guinea-pigs against diphtheria toxin. The observations of Bock and Grossmann (1936) also lent support to the view of the antitoxic property of vitamin C in diphtheria. They instilled *C. diphtheriæ* into the conjunctiva and found that the eye reaction was least marked in those animals which received an excess of ascorbic acid, and most marked in scorbutic guinea-pigs.

Although a number of workers claim to have obtained beneficial results in human as well as in guinea-pig diphtheria, by using a mixture of the cortical extract of the adrenals and ascorbic acid (used apparently because of the involvement of the suprarenal glands in diphtheria) (Herbrand, 1935; Bamberger and Zell, 1936; Thaddea, 1937; Schmidt, 1937; and others), the results obtained in the case of human beings with ascorbic acid alone were not promising. Thus Widenbauer and Saretz (1936) still found a positive Schick reaction in patients after administering a high dose of ascorbic acid (1400 mg.) or daily doses of the vitamin (50 mg.) for prolonged periods; yet the intracutaneous injection of a mixture of 0·2 mg. of diphtheria toxin and 50 mg. of ascorbic acid into a Schick-positive patient yielded a negative result. Otto (1936) observed no difference in the condition between two groups of patients who received 500 units of antitoxin per kg. of body-weight with or without the addition of 500–700 mg. of ascorbic acid, in spite of a retention of the vitamin by the patients of the former group.

The belief entertained by some that ascorbic acid possesses antitoxic properties in diphtheria is, therefore, based on the three following main

observations: (*a*) that in guinea-pigs suffering from diphtheria there is a marked diminution in the ascorbic acid content of the adrenals, (*b*) that ascorbic acid destroys the activity of diphtheria toxin *in vitro*, and (*c*) that guinea-pigs receiving generous quantities of vitamin C are more resistant to the effect of diphtheria toxin than animals which are deficient in ascorbic acid. The first two reasons can be dismissed as unconvincing, since the depletion of the adrenals and *of other organs* of vitamin C is not specific to diphtheria, but may be traced to various causes, and is in all probability secondary in nature. Nor is the destructive action of ascorbic acid on diphtheria toxin *in vitro* a proof that the vitamin possesses antitoxic activity; many substances inactivate diphtheria toxin *in vitro* without being able to exercise antitoxic action *in vivo*. Whether the deleterious effect of ascorbic acid on diphtheria toxin *in vitro* is due to its acidity, as maintained by some workers, or to its reducing property as maintained by others, is irrelevant to the present issue. If, however, ascorbic acid can, as it is claimed, modify the action of diphtheria toxin *in vivo*, the problem becomes of great interest. In the experiments to be described this claim could unfortunately not be substantiated.

TECHNICAL.

The guinea-pigs, which belonged to a vigorous stock, were kept during the pre-experimental period on a diet of oats, bran and fresh cabbage *ad lib.* About 40–50 g. per day of the latter, which is roughly equivalent to 40–50 mg. of ascorbic acid, was consumed by each guinea-pig. All the animals were vaccinated with a mixture of *Salmonella enteritidis* (Gaertner) and *S. typhi murium* (Aertrycke), and only those which were making very good growth were used. The diphtheria toxin, for which I am indebted to Dr. G. F. Petrie, of the Lister Institute, was injected subcutaneously in the abdominal region. The ascorbic acid doses, which were dissolved and partially neutralized immediately before use, were injected into the femoral muscle. When the injection of the vitamin was continued for several days the doses were introduced into the femoral and biceps muscles in rotation in order to avoid local discomfort. In the case of the guinea-pigs which received the pre-experimental mixed diet or extra quantities of ascorbic acid, the oats, bran and cabbage *ad lib.* were continued. During the first day after the injection the animal usually consumed almost as much cabbage as during the pre-experimental period, but as the disease developed the daily consumption of the cabbage diminished; it, however, never fell below an equivalent of 5–10 mg. of ascorbic acid. The guinea-pigs which were depleted of vitamin C were kept for 5 or 6 days before injection on a scorbutic diet which consisted of bran 6 parts, barley meal 2 parts, middlings 3 parts, fish meal 1 part, crushed oats 4 parts by volume, and no more than 60 ml. of autoclaved milk made up from a dried powder. This diet was in their case continued to the end of the experiment.

RESULTS.

No full appreciation of the results can be obtained until the general response of the guinea-pig organism to the administration of various quantities of vitamin C is brought into focus. When these animals are maintained on a

mixed diet such as the above they become " saturated " with the vitamin, that is to say the ascorbic acid content of the tissues reaches a concentration which cannot be raised by a further increase in the oral consumption of vitamin C. In this condition certain tissues, such as the adrenal (about 1·5 mg./g.), the small intestine (about 0·15 mg./g.), liver (about 0·25 mg./g.), contain much more than the bulk of the tissue such as the muscle and bone (about 0·04 mg./g.). The blood-plasma content is in this case on the average only about 0·5 mg. per 100 ml., if, which is probably the case, its very slight indophenol-reducing capacity be taken to be due to the presence of ascorbic acid. The condition of " saturation " persists only above a certain level of intake of vitamin C. Below this level the tissue content of ascorbic acid shows a tendency to diminish, and may even almost disappear in animals which are receiving daily doses as high as 5–6 times the minimum dose necessary to protect them from scurvy. Another striking feature is that the body-reserve of vitamin C of a fully " saturated " guinea-pig practically disappears after the animal is maintained on a scorbutic diet for only five or six days. Animals so depleted of vitamin C are then found to be in excellent health, to grow normally, and to be free from scurvy ; no ascorbic acid can, of course, be detected in their plasma. Although the vitamin C content of the plasma even of " saturated " guinea-pigs is very low, this can, nevertheless, be raised considerably at will, by injecting large doses of ascorbic acid. During the first few hours after injection high quantities of the injected vitamin are circulating in the blood, in spite of the tendency of the animal to excrete this excess quickly in the urine. The following representative experiment will illustrate the point : A series of guinea-pigs of about 250 g. in weight which were previously kept on a mixed diet with cabbage *ad lib.* were injected with 50 mg. of *l*-ascorbic acid. Animals were killed $\frac{1}{2}$ hour, 1 hour, 2 hours, $2\frac{1}{2}$ hours and 4 hours after the injection, and the ascorbic acid was determined in the plasma. The figures obtained were 32·5 mg., 17·5 mg., 11 mg., 7·5 mg. and 3·5 mg. per 100 ml. of plasma respectively.

The results of all the experiments are summarized in the table. The data have not been arranged chronologically but, for convenience, in ascending order of the quantities of vitamin C consumed by the various groups of guinea-pigs. Even a cursory glance at the figures makes it clear that the resistance of the animals to the diphtheria toxin was the same and independent of the level of intake of vitamin C. In experiments D and E (see table) one group of the animals was depleted of vitamin C and consequently their tissues, including the blood, contained very little, if any, ascorbic acid. On the other hand, the other group which was on diet containing cabbage *ad lib.*, *i. e.* consuming *per os* an equivalent of about 10–40 mg. of ascorbic acid per day, received in addition 250 mg. of ascorbic acid by injection twice daily for $2\frac{1}{2}$ days after the introduction of the toxin. The figures given above will indicate the very high concentrations of ascorbic acid in the plasma which were reached under these circumstances. Such concentrations could not be attained from the diet, however rich in vitamin C, even if it be assumed that under conditions of stress all the ascorbic acid contained in the tissues is thrown into the circulation. In spite of this abnormally high ascorbic acid content of the plasma the injected animals did not show any greater resistance to the toxin than did the

Expt.	Diet.	Mg. *l*-ascorbic acid injected.	M.L.D.	Weight (g.). Initial.	Weight (g.). Final.	Survived days.
D	Vitamin C-depleted for 5 days on scorbutic diet	None	1	325	272	3
			1	305	285	2–3
			1	330	262	4
			1	330	315	3
			1	350	255	10*
			1	310	265	10*
E	Vitamin C-depleted for 6 days on scorbutic diet	,,	1	225	202	4–5
			1	255	220	11
			1	250	200	11*
			1	242	185	6–7
			1	240	170	11*
			1	245	202	4–5
B	Mixed diet	,,	1	250	217	3–4
			1	245	200	4–5
			1	255	255	2
C	,,	,,	1·5	250	265	1–2
			1·5	255	250	3
			1·5	257	255	2–3
A	,,	,,	2	260	257	1–2
			2	255	237	2
A	Mixed diet	50 About 10 mins. before injection of diphtheria toxin	2	250	250	1–2
			2	255	240	1–2
			2	255	240	2
			2	252	257	1
B	,,	75 Just before injection of diphtheria toxin	1	250	225	3–4
			1	253	262	2–3
			1	245	205	4–5
C	,,	,,	1·5	250	235	3–4
			1·5	250	240	2
			1·5	250	227	2
D	Mixed diet	250 in 50-mg. doses twice daily, morning and afternoon. First dose injected 15 mins. after diphtheria toxin	1	310	325	2
			1	325	272	4
			1	305	285	4–5
			1	330	305	5–6
			1	330	305	3–4
			1	350	285	10*
E	,,	,,	1	250	245	9*
			1	250	225	4–5
			1	247	205	4
			1	242	195	9*
			1	252	215	9*
			1	242	200	5–6

* Killed by chloroform.

guinea-pigs which were depleted of the vitamin. It is, therefore, not surprising that the animals on the mixed diet with or without single injections of ascorbic acid reacted similarly. These results, which are clear-cut, do not support in any way the contention that vitamin C possesses antitoxic properties against diphtheria toxin.

SUMMARY.

Experiments are described which do not support the view that vitamin C has a protective action against diphtheria toxin in guinea-pigs.

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FURTHER STUDIES OF THE AGENT OF THE ROUS FOWL SARCOMA: A. ULTRA-CENTRIFUGATION EXPERIMENTS; B. EXPERIMENTS WITH THE LIPOID FRACTION.

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A. ULTRA-CENTRIFUGATION EXPERIMENTS.

THE technique of adsorption and elution with aluminium hydroxide was originally designed by Wilstätter for the isolation of enzymes. It was first used by Fraenkel (1929) for the isolation of the agent of the Rous fowl sarcoma and its successful use in this way was believed to prove the enzymatic nature of the agent. As, however, the same method was subsequently used for the